

Repertoire Cloning of Lupus Anti-DNA Autoantibodies

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Abstract

To investigate the autoantibody repertoire associated with SLE, we have created phage display IgG Fab libraries from two clinically active SLE patients and from the healthy identical twin of one of these patients. The libraries from the lupus discordant twins were found to both include unusually large representations of the V_H5 gene family. By panning with DNA, the SLE libraries each yielded IgG anti-double-stranded (ds) DNA autoantibodies, which are characteristic of lupus disease. These included a V_H5 autoantibody from the affected twin, that has a targeted cluster of mutations that potentially improves binding affinity. The recovered IgG anti-dsDNA autoantibodies expressed the same idiotypes associated with the in vivo IgG anti-dsDNA response of the respective SLE donor. Heavy-light chain shuffling experiments demonstrated a case in which the in vitro creation of anti-dsDNA binding activity required restrictive pairing of a heavy chain with V_λ light chains similar to those in circulating anti-dsDNA autoantibodies. By contrast, IgG anti-ds autoantibodies could not be recovered from the library from the healthy twin, or from shuffled libraries with heavy chains from the healthy twin. These repertoire analyses illustrate how inheritance and somatic processes interplay to produce lupus-associated IgG autoantibodies. (*J. Clin. Invest.* 1996. 98:2827–2837.) Key words: lupus • repertoire • autoantibody • anti-DNA • phage display

Introduction

High levels of circulating autoantibodies (autoAbs)¹ are a hallmark of SLE, and animal models have confirmed that disease and premature death occur only after the skewing of the B cell repertoire toward autoreactivity (1, 2). While the pathophysi-

ology of SLE is complex, there is much evidence to suggest that pathogenic autoantibodies are largely responsible for the organ damage, particularly the renal disease that is the primary cause of disability and death (3).

Circulating antibodies (Abs) that react with double-stranded (ds)DNA in vitro are diagnostic and prognostic markers for SLE. They also correlate with the development of lupus nephritis, especially diffuse proliferative glomerulonephritis (4, 5). While recent studies suggest that not all anti-dsDNA autoAbs are nephritogenic (reviewed in references 6 and 7), there is still considerable evidence that the transfer of certain anti-dsDNA autoAbs into healthy mice results in kidney deposition and renal damage (8, 9). Investigations of the role of disease-associated autoAbs in the pathophysiology of murine models of lupus have generally been much more successful than human studies. In large part, this is because human monoclonal autoAbs, and in particular the IgG autoAbs that are often implicated in organ damage, have been difficult to obtain from human donors using available cellular technologies. In addition, while autoimmunity-prone strains of mice display certain features of human disease, it is increasingly apparent that these mice also have idiosyncrasies that are not relevant to human pathophysiology.

In recent years, an alternative means for sampling an Ab response, known as repertoire cloning (10), has been developed. This approach enables the generation of mAbs of defined specificity, by the molecular cloning of expressed Ab heavy (H) and light (L) chains genes that are isolated directly from lymphocytes, which are selected based on binding specificity after in vitro expression. We have recently reported our use of repertoire cloning for the recovery of IgG anti-dsDNA autoAbs from a SLE patient (11). We have focused on autoAbs to dsDNA, because of the evidence of their role in SLE pathogenesis, and because there is a large body of data regarding these autoAbs from murine and human B cell lines that enable comparisons regarding genetic, immunochemical, and functional features.

Our previous report included a library from an active lupus patient, SI. ELISA, surface plasmon resonance determinations, and *Crithidia* immunofluorescence assays documented that high affinity IgG autoAbs to dsDNA can be recovered from a phage-display Fab library made from the peripheral blood lymphocytes of an SLE patient (11).

In the current study we extend these studies to include additional IgG Fab libraries constructed from the peripheral blood lymphocytes of a set of identical twins discordant for SLE. By panning on human dsDNA, IgG anti-dsDNA autoAbs could be recovered only from the library of an SLE patient with high circulating levels of this type of autoAb, and not from a library from the healthy identical twin of one of these patients. Using antigen (Ag)-binding assays, tests for three different cross-reactive idiotypes, V region DNA se-

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1. Abbreviations used in this paper: Ag, antigen; autoAb, autoantibody; autoAg, autoantigen; H, heavy chain; L, light chain.

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quence analysis and immunoglobulin chain shuffling studies, we characterized the anti-DNA autoAbs obtained from these libraries, and demonstrated their relationship to the autoAbs present in the circulation of the two SLE donors.

Methods

Creation of combinatorial Ig libraries. The two female SLE patients, SI (previously reported [11]) and AA, were diagnosed by the criteria of the American Rheumatism Association (ARA), and used as donors in the generation of combinatorial libraries. These patients had high circulating levels of IgG Abs to dsDNA, and other clinical and serologic features of classical SLE. Patient AA was a nulliparous 26-year-old Caucasian female, who initially presented with malar rash, nondeforming polyarthritis, hypertension, mild renal insufficiency, proteinuria, active urinary sediment, anemia, and thrombocytopenia. She developed biopsy-proven diffuse membranoproliferative lupus nephritis that progressed to complete renal failure shortly after sample donation. AA had shared an amniotic sac and placenta with an identical twin, BB, who was gravida 2, para 2, had no history or clinical findings consistent with SLE or other active medical problems. Despite a high titer antinuclear Abs, BB did not have significant levels of IgG autoAbs to dsDNA. Neither of the twins had detectable anticomplement, Sm, RNP, or rheumatoid factor autoAbs. After informed consent, peripheral blood from these subjects was obtained as the source of mRNA for generation of IgG1 libraries, the predominant subclass of lupus anti-dsDNA Abs (12). As previously described (13–16), PCR was used to rescue and clone Ab genes from human tissue into the phage display vector, pCOMB3H (gift of Dr. C.F. Barbas, The Scripps Research Institute, La Jolla, CA), to construct each library. In a library, Ab genes are cloned within the phage-mid vector, and surface display phagemid clones are then selected based on Ab binding specificity. To construct the AA and BB libraries, a previ-

ously described panel of V_H and V_L first framework region specific primers (16) were supplemented with primers to ensure representation of newly reported V_λ genes: VL1b/5, 5'-CAG TCT GAG CTC ACT CAG CCA CC-3'; VL9, 5'-CAG TCT GAG CTC ACT CAG CCT CCC-3'; VL3', 5'-TCC TAT GAG CTC ACT CAG CCA CCC-3'; VL8' 5'-CAG CTT GAG CTC ACT CAA TCG CCC-3'; VL7, 5'-CAG GTT GAG CTC ACT CAA CCG CCC-3'. The PCR conditions have previously been described (15, 16). In an effort to make comparable libraries, equal amounts of the 660–700 bp DNA band from each amplification was included in the AA and BB libraries. The resultant libraries contained > 2 × 10⁷ colony forming units (cfu). Individual pCOMB3H clones were designated according to its library/donor source (SI, AA, or BB).

Antibody selection. To avoid loss of phage-Fabs through binding to bacterial DNA (or RNA) in the cultures, 2 mg of bovine pancreatic DNase (Sigma Chemical Co., St. Louis, MO), 2 mg of Type II-A RNase (Sigma Chemical Co.) and hydroxyapatite (Sigma Chemical Co.) were included in the medium for phage growth before precipitation and purification (11). As previously described, for selection of DNA-specific clones, the SI library was panned against human placental DNA (Sigma, Chemical Co.) which contains both ss and dsDNA (11), while the AA and BB libraries were panned against placental DNA optimized for dsDNA content (see below). DNA was immobilized on microtiter wells by coating at 10 μg/ml in PBS and air drying. Before use, wells were rinsed twice with dH₂O and blocked with 3% BSA-PBS. For each round of selection, two wells were filled with 50 μl of phage library containing 10¹¹ cfu in PBS, and incubated for 2 h at 37°C. After wells were extensively washed, binding phage were eluted with glycine-HCl, pH 2.5, and rapidly neutralized with 2 M Tris-base. Phage were amplified and rescued prior to each round of panning.

Chain shuffling experiments. The AD4-37 anti-DNA Ab is representative of one the two major Ab sets selected from the IgG Fab κ/λ library from the SLE-affected twin, AA, after four rounds of selec-

Table I. Idiotypic Reactivities and Structural Homologies of Recombinant Lupus IgG Anti-dsDNA Autoantibodies

	V _H gene usage						V _L gene usage			
	18/2 CRI*	B6 CRI	V _H family	Nearest V _H [‡]	V _H DNA	J _H	VλIIIa CRI	V _L family	Nearest V _L	V _L DNA
AA library										
AD4-37	–	+	3	1.9III/V3-30	99.3	J _H 3b	+	λ3	III.1	99.2
AD4-18	–	–	5	V _H 251/V5-51	91.9	J _H 5a	–	λ2	Ialh2*	85.4
AA H-L chain shuffled libraries										
With AD4-37V _L [§]										
AAD4-HC2	–	–	3	Cos 6/V3-74	92.9	J _H 3b	+	λ3	III.1	99.2
AAD4-3/3	–	–	3	p1	88.2	J _H 5b	+	λ3	III.1	99.2
With AD4-37V _H										
ABD4-lam2	–	+	3	1.9III/V3-30	99.3	J _H 3b	–	λ2	DPL13	93.6
ABD4-lam3	–	+	3	1.9III/V3-30	99.3	J _H 3b	–	λ1	IGLV1S2	99.3
SI library [¶]										
SI-32	+	–	3	V _H 26/V3-23	97.8	J _H 4b	–	λ3	IGLV3S1	99.6
SI-13	+	–	3	V _H 26/V3-23	91.6	J _H 4b	–	λ2	DPL1	96.1
SI-1	+	–	3	GL-6	97.8	J _H 4b	–	κIII	kv325	97.6
SI-40	–	+	3	Cos3	98.9	J _H 6b	–	λ1	lv1042	95.7
SI-22	–	+	3	Cos3	97.8	J _H 6b	–	λ1	lv1042	98.8
SI-39	–	–	1	DP-75	94.9	J _H 6b	–	κIII	kv325	97.6

*CRI expression were determined by direct binding and inhibition ELISA. [‡]The closest known V_H or V_L germline gene is indicated. Calculations of homology omit the contribution of the upstream FR1 primer. [§]The clones that begin with AAD were selected using the V_L region of the AD4-37 clone and a library of H chain genes from the affected twin, AA. The clones that begin with ABD were selected with the V_H region of AD4-37 and a library of L chain genes from the healthy twin, BB. ^{||}Cos6 is an allele of V3-74 (I. Tomlinson, personal communication). [¶]The SI library and these DNA antibodies have previously been described (11).

tion with human placental dsDNA (Table I). To evaluate the relative contribution and/or permissiveness of the H and L chains of the AD4-37 Ab for the DNA binding specificity, four additional shuffled libraries were created. Using the same restriction sites used for cloning, a shuffled library was created in which the H chain gene of AD4-37 replaced all H chain genes paired with the BB- κ (healthy twin's) library of diverse L chains. We created a separate shuffled library by pairing this same H chain gene of AD4-37 with the BB- λ library of diverse L chains. A third library was created, in which the L chain gene of AD4-37 was paired with the BB library of diverse V_H -gamma rearrangements. A fourth library was also created in which this same L chain gene of AD4-37 was paired with the AA (affected twin's) library of diverse V_H -gamma genes. We also attempted to create a library in which the H chain gene of AD4-37 replaced all of the H chain genes paired with the AA library of diverse V_L gene rearrangements, however multiple attempts at phage selection with dsDNA-coated wells yielded only clones with deleted L chain genes.

Colony hybridization to survey of V_H family diversity. To survey V_H chain diversity within the pComb3H clones, the AA and BB libraries were screened by hybridization with V_H family-specific framework region derived 30–40-mer oligonucleotides, as previously reported (16), and with oligonucleotides from the CDR1 of the AD4-37 V_L , 5' GAT AAA TTG GGG GAT AAA TAT 3'; the CDR3 of the AD4-18 V_L , 5' CCA GTT GTA CCG CCC GGG TAG T 3; and the CDR3 of the AD4-37 V_H , 5' CCA AAG CCC TCG GTA CTA TCA T 3'.

DNA sequence determination. The nucleotide sequences of the V_H and V_L regions were determined by automated sequencing (Applied Biosystems, Foster City, CA), and each was determined at least three times using two different primers with previously described primers (15, 16). Analyses used Macvector 4.0 (IBI, New Haven, CT) and GCG (Madison, WI) software packages, with comparison to GENBANK and the V Base Directory.²

Antibody binding assays. The (ds)DNA binding activity of serum and cloned Abs was evaluated in an ELISA format that produces results that have been correlated with *Crithidia lucilae* immunofluorescence and surface plasmon resonance determinations of native DNA binding (11). High molecular weight human placental DNA was used (17). Because immobilization of the dsDNA on the solid phase may expose determinants associated with ssDNA, all Abs were evaluated using both ssDNA and dsDNA precoats to better assess binding specificity (11). For these assays, dsDNA and ssDNA were prepared as previously described, and microtiter wells (Cat. No. 3690; Costar Corp., Cambridge MA) were coated with 0.5 μ g of DNA per well in 50 μ l water and dried overnight. Wells were washed 10 times with 0.05% Tween-BBS and blocked for 1 h at 37°C with 3% BSA in PBS. Wells were then emptied and filled with serum samples diluted in 1% BSA-PBS and incubated for 2 h at 37°C. Wells were then washed 10 times with Tween 20-BBS, and incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated affinity purified goat anti-human IgG or IgM (Boehringer-Mannheim, Indianapolis, IN) diluted 1:2,000 in 1% BSA-PBS. Plates were washed and developed with the TMB substrate (Kirkegaard and Perry Labs, Gaithersburg, MD) and the OD (450–650 nm) determined. For other binding assays, BSA, methyl-BSA, actin, cardiolipin, ovalbumin, dextran sulfate (Sigma Chemical Co.), tetanus toxoid (Connaught Labs, Swiftwater, PA), or human IgG Fc fragments (Jackson Labs, West Grove, PA) were substituted using the same conditions. In all studies, samples were designated as positive if they were at least three SD above highest value obtained for 32 healthy adult controls.

Idiotypic assays. The murine IgG1 mAb, B6, identifies the products of genes and alleles related to only two of the \sim 20 functional germline V_H3 gene segments (18)(V Base Sequence Directory),

which include the 1.9III/V3-30 and Cos3 genes ([19] and G.J. Silverman, unpublished observations). The anti-18/2 CRI, is identified by the absorbed rabbit serum, termed Ra504, which was induced by immunization with 18/2, a human IgM anti-ssDNA Ab encoded by an unmutated $V_H26/V3-23$ gene (20–22). An idiotype expressed on mAb 18/2 has previously been associated with lupus autoAbs with anti-DNA and other self-specificities (22, 23). The murine IgG_{2b} mAb, termed 22-15D11 or λ IIIa, recognizes a subset within human $V_{\lambda}III$ L chains (24), and this idiotype was associated with an anti-IgG autoAb (rheumatoid factor) from a rheumatoid arthritis patient (25).

Assays of idiotype expression were designed to identify the idiotype expressed on serum IgG autoAbs, and avoid detection of the idiotype on the parallel set of Abs that do not possess autoreactivity. In a sensitive direct binding ELISA, microtiter wells were coated overnight at 4°C with highly purified dsDNA at 10 μ g/ml, blocked with 3% BSA in PBS, and incubated for 4 h at room temperature (RT) with either the human monoclonal Fab at 1 μ g/ml, or a serum that was serially diluted beginning at 1:100. Thereafter, wells were washed and incubated for 1 h at 37°C with the antiidiotype or an equivalent concentration of an isotype control, then washed and incubated with the appropriate HRP-labeled anti-rabbit IgG or mouse IgG (Boehringer-Mannheim Biochemicals), then developed.

Reactive sera were evaluated in inhibition studies, in which sera at a fixed dilution (1/800) in the linear portion of the curve were preincubated for 1 h with a saturating concentration (up to 40 μ g/ml) of the antiidiotypic Ab (or an isotype control). These samples were subsequently incubated in dsDNA precoated plates for 4 h at RT. Plates were washed and binding was detected with HRP-tagged anti-human IgG, IgM, κ or λ Ab (Tago, Burlingame, CA). Serial dilutions of the same serum without inhibitor were used for quantitation. For direct antiidiotype assays, wells were instead precoated with an antiidiotypic Ab at 10 μ g/ml, then developed as described above.

Results

Selection and characterization of autoantibodies from phage display libraries. Human placental dsDNA was used as the selection Ag for Abs from the IgG Fab library from each twin. Significantly, anti-dsDNA Abs were successfully selected from the library from the affected twin, AA (Fig. 1) but despite multiple attempts using the same conditions we were unable to se-

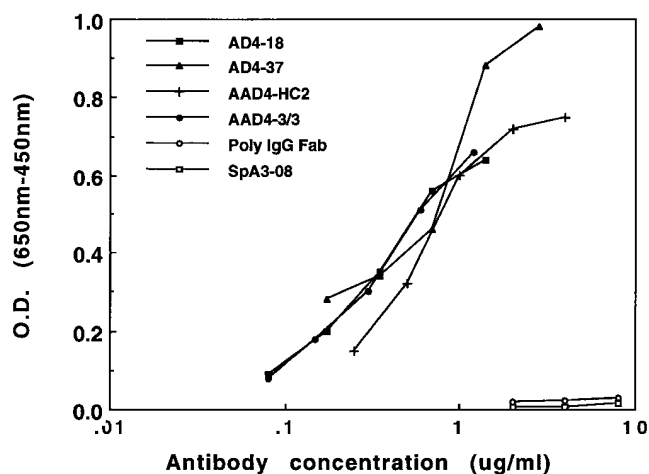


Figure 1. The DNA binding activities of select autoantibodies. Binding activities of the purified monoclonal IgG Fab for dsDNA pre-coated ELISA plates are compared with adult polyclonal IgG Fab (*Poly IgG Fab*), and a control monoclonal IgG Fab (*SpA3-08*)(15). The binding activities of the autoAbs from the SI library have previously been reported (11).

2. A Database of Human Immunoglobulin Variable Region Genes. I.M. Tomlinson, S.C. Williams, S.J. Corbett, J.P.L. Cox, and G. Winter. MRC Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, United Kingdom.

	FR1	CDR1	FR2	CDR2	FR3
GL 1.9III AD4-37	QVQLV ES GGG VVQ PGR SLRLS CAAS GFTFS L	SYGMH	WVRQAPGKGLEWVA	VISYDGS NK YYADSVKG C	RFTISRDN SKNT LYLQ MNSL RAEDTAVYYCAK I S
GL COS-6 AAD4-HC2	EVQLV ES GGG L VQ PGGSLRLS CAAS GFTFS L C S	SYWMH D Y	WVRQAPGKGLVWVS T Q	RINSDGSSTSYADSVKG R	RFTISRDN AKNT LYLQ MNSL RAEDTAVYYCA I
GL p1 GL AAD4-3/3	EVQLV ES GGG L VQ PGGSLRLS CSAS GFTFS L L	SYAMH NF I	WVRQAPGKGL EYVS Q C	AISS MG S TY YADSVKG G I R	RFTISRDN SKNT LYVQ MSSL RAEDTAVYYCVK Q DVV L G ID A M R
GL VH251 AD4-18	EVQLV QSGAEVKKP GES LKIS CKG SGYSFT L E V G	SYWIG N	WVRQMPGKGLEWVG V	I Y PGDSD TR YS PS FQ N	QVTISADK SISTAYLQ WSS LKAS DTAMYYCAR T N V K

Figure 2. V_H regions of autoantibodies. The deduced amino acid sequences of the V_H regions of IgG Fab anti-dsDNA Abs are displayed. Sequences are compared to the closest known germline gene. Identity is demonstrated by a horizontal line, with amino acid differences indicated. These sequence data are available from EMBL/GenBank/DBJ under accession numbers U70232, U70233, U70236, and U70238.

lect Abs with dsDNA binding activity from the library of the healthy twin, BB. Abs with ssDNA binding activity could be derived from both libraries (not shown).

DNA sequence analysis of the V_H regions of 10 randomly selected plasmid clones from the AA library obtained after four rounds of selection on DNA coated wells demonstrated the presence of only two distinct V_H rearrangements, represented by the AD4-37 and AD4-18 mAbs (Table I, Figs. 2 and 3). V gene analysis of these two Abs revealed intriguing structural and genetic features. Strikingly, the H and L chain genes of the AD4-37 Ab are only minimally mutated, differing by only two point mutations from known germline genes, i.e., V_H (1.9III/V3-30) and V_L (III.1), respectively (Figs. 2 and 4, Table I). In an independent isolate we identified a V_H gene rearrangement that was identical except for the incorporation of the sequence of a different upstream FR1 primer, suggesting that the AD4-37V_H sequence variations from the V3-30 germline gene are probably not cloning artifacts. This same germline V_λIII gene and a > 97% homologous V_H3 gene segment are paired in an IgM anti-phospholipid autoAb produced by cellular techniques (26). This exact V_H gene segment in germline configuration, paired with a germline encoded V_λI light chain, was previously identified in an IgM anti-DNA autoAb from a hybridoma (27).

The AD4-18 anti-dsDNA autoAb is encoded by a V_H5 gene with a total of 12 mutations including 7 replacement mutations. Interestingly, this V_H5 gene has a cluster of three point mutations in two adjacent codons in the tip of the FR3 loop that changes the germline isoleucine-serine to newly create threonine-asparagine (Fig. 2), at a mutational hotspot (28).

The L chain of the AD4-18 Ab is most homologous to a V_L gene segment in the V_λII gene family termed DPL7 (29) (Fig. 4).

Surveys of V gene usage in the libraries by colony hybridization. To evaluate independently the molecular diversity in the AA and BB libraries, surveys were performed with V_H gene family (16) and V gene-specific oligonucleotide probes (Table II). The IgG libraries from the twins had almost identical distribution of V_H families. The large V_H1, V_H3, and V_H4 families, which are commonly expressed in the peripheral repertoires of healthy adults (15, 16, 30, 31), were well represented (Table II). However, more than one third of the gamma rearrangements in both the AA and BB libraries derived from the V_H5 gene family, while the V_H5 family is the source of < 1% of the rearrangements in several libraries made from the healthy adults constructed with the same methods ([15, 16] and G.J. Silverman, unpublished observations).

Almost all of the colonies in the AA library obtained following four panning with dsDNA were derived from either the V_H3 or V_H5 families (92%) (Table II), which was consistent with the DNA sequence results (see above). Hybridization studies also confirmed that after panning, the V_HAD4-37, V_LAD4-37, and V_HAD4-18 genes are highly represented (≥ 20%) amongst their respective rearrangements. Compared with the unselected AA library, representation of each of these specific V_H and V_L rearrangements was increased 10- to 14-fold in the dsDNA-selected library (*P* < 0.05, by chi-square analysis).

By contrast, panning with dsDNA did not appreciably change the representation of the different V_H families in the

	VH _{seq}	CDR3	FR4	
			D _H segment	
AAD4-HC2	TGT GC Cys Ala	T TCA AAG GCT ATG ATA GTA CCG AGG Ser <u>Lys</u> Ala Met Ile Val Pro <u>Arg</u>	GCT TTT GAT ATC Ala Phe Asp Ile	TGG GGC CAA GGG ACA ATG GTC AAC GTC TCT TCA Trp Gly Gln Gly Thr Met Val Asn Val Ser Ser
AAD4-3/3	TGT GTG AGA Cys Val <u>Arg</u>	GGC CCT TCG ACG ACT CTA CAT ACT Gly Pro Ser Thr Thr Leu His Thr	AAT TGG TTC GGC CCC <u>Asn</u> Trp Phe Gly Pro	TGG GGC CAG GGA ACC CTG GTC AGC GTC TCC TCA Trp Gly Gln Gly Thr Leu Val Ser Val Ser Ser
AD4-37	TGT GC Cys Ala	T TCA AAG GCT ATG ATA GTA CCG AGG Ser <u>Lys</u> Ala Met Ile Val Pro <u>Arg</u>	GCT TTT GAT ATC Ala Phe Asp Ile	TGG GGC CAA GGG ACA ATG GTC ACC GTC TCT TCA Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
AD4-18 DXP'1	TGT GCG A Cys Ala <u>Lys</u>	AA CTA CCC GGG CGG T Leu Pro Gly <u>Arg</u> Tyr AA CTc CCC G	AC AAC TGG TTC GAC TCC <u>Asn</u> Trp Phe Asp Ser	TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser

Figure 3. V_H CDR3 sequences of anti-dsDNA antibodies. The V_H regions of AD4-37 and AAD4-HC2 are shown to share identical CDR3 and FR4 regions, despite employing distinct V_H3 gene segment contributions. Only for the V_HAD4-18 was significant homology detected with known D_H and DIR genes. The same nonamer sequence in V_HAD4-18 had one nucleotide mismatch the same sequence in dxp'1 and d21.

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
GL DPL7 AD4-18	QSVVTQPPSVSGAPGQRVTISC MAEL	TGSSSMIGAGYDVH P F	WYQQLPGTAPKLLIY I VF	GMSWRPS DWS	GVPDRFSGSKSGTASLAITGLQAEDEADYYC A F S V D	QSYDSSLG T M SVF	FGGGTKLTVL
GL III.1 AD4-37	SVELTQPPSVSVSPGQTASITC MA S V	SGDKLGDKYAC	WYQQKPGQSPVLVIY I	QDSKRPS	GIPERFSGSNGTATLTISGTQAMDEADYYC	QAWDSSTA W VV	
GL DPL13 ABD4-1am2	QSAITQPPSVSGSPGQSVTISC AAE	TGTSSDVGSYMRVS T I T D	WYQQPPGTAPKLMIIY S V	EVSWRPS E	GVPDRFSGSKSGTASLTISGLQAEDEADYYC	SLYTSSTTF S W I W I	
GL DPL7/IGLV1 ABD4-1am3	QSVVTQPPSVSGAPGQRVTISC AAEL	TGSSSMIGAGYDVH	WYQQLPGTAPKLLIY	GMSWRPS W	GVPDRFSGSKSGTASLAITGLQAEDEADYYC	QSYDSSLG SV	

Figure 4. V_L regions of anti-DNA antibodies. The deduced amino acid sequences of the V_L regions of IgG Fab anti-dsDNA Abs are displayed. Sequences are compared to the closest known germline gene. Identity is demonstrated by a horizontal line, with amino acid differences indicated. These sequence data are available from EMBL/GenBank/DBJ under accession numbers U70234, U70235, U70237, and U70239.

BB libraries (Table II). While control studies documented dual V_H/V_L genes cloned and Fab displayed on the surface of phage in these libraries, these pannings of the V_H BB libraries did not select for Abs with significant dsDNA binding activity, and did not select for phagemid clones with related or restricted V_H gene DNA sequences (not shown).

Selection and characterization of autoantibodies from shuffled libraries. To evaluate the relative contribution of particular H and L chains to DNA binding, the Ab V genes of AD4-37 were individually paired with libraries of reciprocal chains (Table III). Panning of the library composed of the V_HAD4-37 gene (originally isolated from the AA library) paired with the diverse κ/λ AA library selected for Abs with dsDNA binding activity. Hybridization studies revealed selection for clones with the V_LAD4-37 CDR3 but also with certain other λ-type L chains (Table III). However, in several panning experiments, no autoAbs with κ-type L chains were selected from this library.

The V_HAD4-37 gene was also paired separately with a diverse κ gene library and a diverse λ gene library from BB, the healthy twin. Repeated attempts at panning the V_HAD4-37 gene-BB diverse κ gene rearrangement shuffled library were completely unsuccessful at recovering Abs with dsDNA bind-

ing activity. In contrast, Abs with dsDNA binding activity were selected from the V_HAD4-37 gene- BB diverse λ gene rearrangement shuffled library. Hybridization studies did not detect the V_LAD4-37 rearrangement in these BB L chain-containing libraries before or after panning (Table III). Sequence analysis of individual anti-dsDNA autoAb clones demonstrated selection of other L chain rearrangements exemplified by ABD4-lam3, which derives from the V_λI family, and the ABD4-lam2 (Fig. 4 and Table III) that derives from the V_λII gene family associated with the lupus idiotype 8.12 (32). These studies suggest that the creation of dsDNA binding activity can only occur when the H chain from the V_HAD4-37 gene product is paired with a L chain from one of a limited set of V_λ gene rearrangements.

In the reciprocal chain shuffling studies, the V_LAD4-37 gene was paired with the diverse V_H gene rearrangement library from the affected SLE twin, AA. Again, panning successfully selected for Abs with dsDNA binding activity, and hybridization studies confirmed enrichment for the original partner gene V_HAD4-37 (Table III). Sequence analysis confirmed this selection, and also demonstrated an autoAb Fab clone, termed AAD4-HC2, that uses a distinct V_H3 gene that appears to be derived from a separate germline gene segment,

Table II. DNA Hybridization Studies of Twin IgG Fab Libraries

	V _H 1*	V _H 2	V _H 3	V _H 4	V _H 5	V _H 6	AD4-37V _H CDR3	AD4-18V _H CDR3	AD4-37V _L CDR1 [‡]	AD4-37V _L CDR3
AA libraries [§]										
Unselected	11	0	10	31	42	< 1	2.4	1.4	5	2.4
Selected	1.5	0	34	4	31	0	30	20	22	25
BB libraries										
Unselected	16	0	11	31	37	< 1	1.8	< 1	2	2
Selected [¶]	14	0	7	36	32	< 1	< 0.5	< 1	4	< 0.5

*These data represent results of hybridization of membrane lifts of bacterial colonies with 150 to 250 colonies per plate for each V_H gene probe, with subsequent stripping and reprobing with a constant region-specific probe. Data are presented as percentages of colonies that also hybridized with a C_γ-specific probe. Controls included in each study were plasmids with V_H genes of all known families, or AD4-18 and AD4-37 plasmids. In each V_H region survey certain colonies were unidentified, either because they did not contain V_H genes or presumably because sequence variations abolished hybridization. Confirmation of reproducibility of values was confirmed by performance of each hybridization study at least twice. [‡]The values for the AD4-37V_L-derived oligonucleotide hybridizations were calculated as the percentage of colonies that also hybridized with a C_λ-specific probe. Studies with a CDR1 sequence probe from V_LAD4-37 demonstrated the same representation detected with the CDR3 probe from V_LAD4-37. [§]The selected libraries were obtained after four sequential rounds of panning on wells precoated with dsDNA. Studies with oligonucleotide probe for the AD4-37V_H rearrangement, AD4-37V_HCDR3, display > 12-fold enrichment in the selected AA library, and > 14-fold enrichment with the AD4-18V_H rearrangement probe, AD4-18V_HCDR3. The oligonucleotide probe for the AD4-37V_L rearrangement, AD4-37V_LCDR3, displays 10-fold enrichment in the selected AA library. ^{||}These values in the AA selected library are statistically significant in relation to the unselected library (P < 0.05) as determined by chi-square analysis, with the BB libraries used for the comparisons. [¶]Hybridization studies of the BB library after four rounds of panning against dsDNA did not detect a change in the V_H family clonal distribution.

Table III. DNA Hybridization Studies of Shuffled IgG Fab Libraries*

	AD4-37V _H CDR3*	AD4-37V _L CDR3
V _H 4-37 with AA L chain library		
Unselected		2
Selected		9
V _H 4-37 with BB L chain library		
Unselected		<< 1
Selected		<< 1
V _L 4-37 with AA H chain library		
Unselected	0.8	
Selected	6.4	
V _L 4-37 with BB H chain library		
Unselected	<< 1	
Selected	0	

*In each shuffled library, a H or L chain gene from the AD4-37 clone was paired with a diverse library of genes for the reciprocal chains from either the AA or BB library, as indicated. Hybridization studies were performed before (*unselected*) and after four rounds of panning with dsDNA (*selected*). In libraries with diverse chains from the AA library, panning enriched for representation of clones reactive with the probe for the original chain mate in the AD4-37 clone, while similar enrichment was not detected with pairing of genes from the BB library. Studies of shuffled libraries were performed and data compiled as described in Table III.

Cos6, but with a DNA sequence for the V-D-J rearrangement that is identical to that of V_HAD4-37. While it is uncertain whether this is an artifact that is due to “jumping” PCR, the selection of this rearrangement supports a central role for the V_HCDR3 subdomain in determining dsDNA binding activity (see below). Within this selected library, clones represented by another V_H3 rearrangement, exemplified by the AAD4-3/3 Fab clone, were also recovered (Figs. 2 and 3).

A shuffled library of the V_LAD4-37 gene paired with the diverse V_H gene rearrangement library from the healthy twin, BB, was also created. However, despite recurrent attempts, panning consistently failed to select for Abs with dsDNA binding activity, although ssDNA binding activity was often present (not shown)(G.J. Silverman, manuscript in preparation). These studies suggest that dsDNA binding activity stringently requires appropriate V_H rearrangements that were not prevalent in the expressed repertoire of the healthy twin.

V_H CDR3 sequence analysis of anti-DNA antibodies. Analyses of the CDR3 amino acid sequences of the dsDNA selected autoAbs demonstrated patterns previously demonstrated in anti-DNA autoAbs recovered from B cell lines. In Fig. 3, these V_H CDR3 all included arginines, and lysines and asparagines were also prominently represented, which have all previously been shown to contribute to DNA binding affinity (reviewed in reference 33). Only in the case of the AD4-18 could we detect homology with a D_H segment (Fig. 3), which are often difficult to distinguish in adult human V_H rearrangements (34).

V_L sequence analysis of anti-DNA antibodies. The V_λ domains of all the cloned anti-dsDNA Abs included arginines and/or asparagines in at least two of the three CDR (Fig. 4). In

κ-type autoAbs, these same amino acids contribute to DNA binding specificity and affinity (35), however the role of V_λ regions in DNA binding has not been systematically examined (36).

Anti-DNA antibodies, cross-reactivity, and fine binding specificity. Fab were evaluated for binding activity with ssDNA, dsDNA, and a panel of other types of Ags (Fig. 5). The AD4-37 autoAb has virtually equal binding interactions with dsDNA and ssDNA (Fig. 5); it also binds dextran sulfate, ovalbumin, tetanus toxoid, and methyl-BSA, and the autoAg IgG Fc, but it does not bind cardiolipin or actin. This pattern of polyreactivity is also displayed by the anti-DNA Ab, KIM4.6, which has a 98% homologous V_H gene segment, and is also paired with a germline λ-type L chain (37). In contrast, the AD4-18 autoAb has the reactivity pattern most commonly associated with high affinity IgG autoAbs (38). It binds to dsDNA and ssDNA near equally, and does not significantly react with other Ags.

The Abs from the shuffled libraries, ABD4-lam2, ABD4-lam3, include the H chain of AD4-37 each with a different L chain. This alternate L chain pairing was associated with a pattern of binding similar to AD4-18, in which the dsDNA and ssDNA are recognized near equally while the other Ags were recognized weakly or not at all. The other Abs from the shuffled libraries, AAD4-HC2 and AAD4-3/3, include the L chain of AD4-37 each with a different H chain. These Abs display the same monoreactivity with dsDNA and ssDNA, with little or no binding with the most of the other Ags, except that AAD4-3/3 has stronger binding with cardiolipin.

Idiotypic expression on monoclonal anti-DNA antibodies. Based on the V gene usage of the IgG anti-DNA autoAbs obtained from the AA library and previously described SI library (11), studies were performed to evaluate the expression of three well characterized anti-idiotypic Abs specific for the V gene segments expressed within these autoAbs (Table I). The murine IgG₁ mAb, B6, identifies the products of only ~ 10% of known potentially functional germline V_H3 gene segments (18, 19). These B6 CRI-associated genes include the 1.9III/V3-30 gene, that encodes the V_H AD4-37 from the AA twin library, and the highly homologous Cos3 gene that encodes the V_HSI-22 and V_HSI-40 anti-DNA Abs from the SI lupus library (Fig. 2 and Table I)(11).

As predicted by V gene usage, two nonoverlapping sets of the cloned IgG anti-dsDNA autoAbs expressed either the B6 or the 18/2 CRI (Table I). Confirming a recent report (25), the serologic reagent, murine monoclonal λIIIa, which recognizes a set within V_λIII L chains (26), reacted with the AD4-37 Ab that has a L chain encoded by a near germline V_λIII.1 gene segment.

Serologic surveys of donors and other SLE patients. High titer (> 1:640) antinuclear Abs were detected in both twins. However only the affected twin, AA, had Abs to native DNA as detected in a *Crithidia* assay (data not shown). In contrast, repeated serial serologic evaluations of the healthy twin, BB, have consistently failed to detect autoAbs reactive by *Crithidia* immunofluorescence. Of the sera from 36 SLE patients evaluated in a sensitive direct binding ELISA, 16 SLE patients (including the donors AA and SI) had significantly elevated levels of IgG dsDNA binding activity (Table IV). The elevated autoAb levels in these SLE patients were also above that detected for all 32 healthy controls. Of the SLE patients, the affected twin, AA, had one of the highest levels of IgG anti-dsDNA activity. Of interest, the level of IgG anti-ssDNA

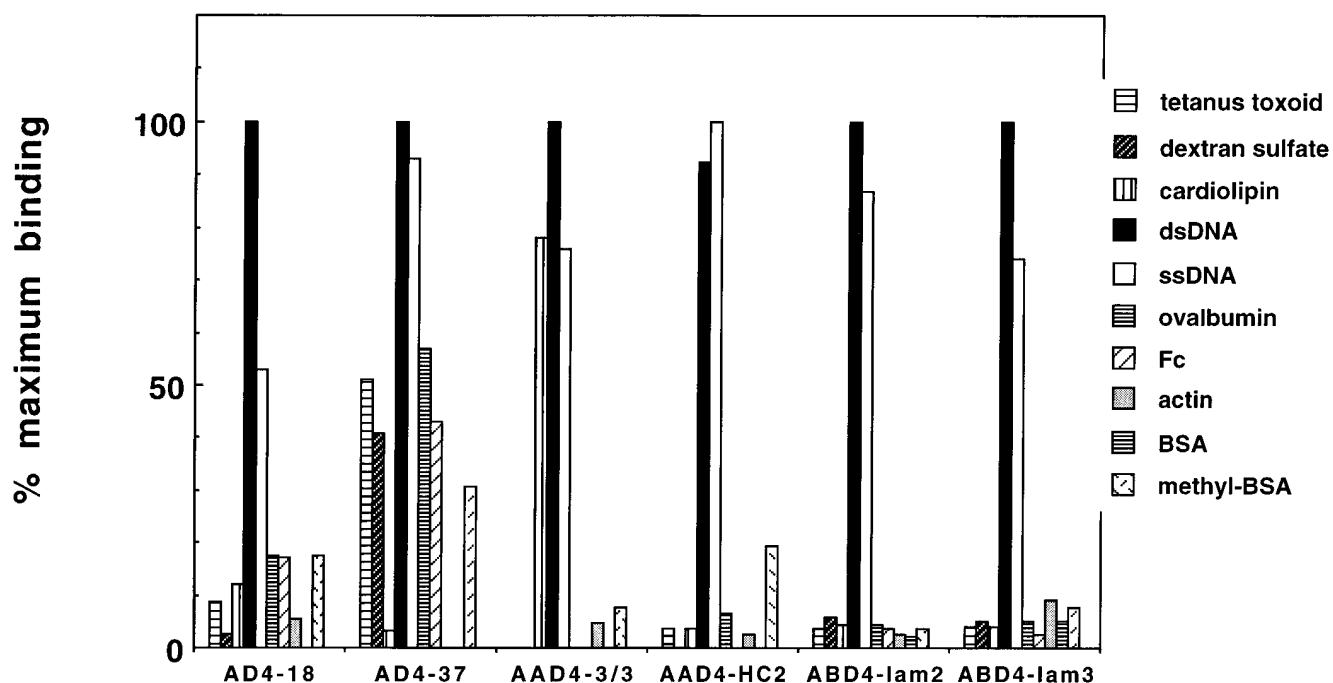


Figure 5. Cross-reactivities of Fabs to a panel of solid-phase antigens tested by ELISA. Reactivities are compared for each monoclonal IgG Fab at 2 μ g/ml. Values are reported as a percentage of the maximum absorbance attained, as previously described (11).

binding in the serum of the healthy twin was also above the range detected in the healthy controls (Table IV).

Idiotypic surveys of the SLE patients and normal controls. Assays of idiotype expression were designed to enable identification of the idiotype expressed on IgG autoAbs in the serum, but to avoid detection of the idiotype on the parallel set of Abs that do not possess autoreactivity. Of the 16 SLE sera with elevated levels of IgG anti-dsDNA Abs, six expressed the B6 CRI, four expressed the λ IIIa CRI and 10 expressed the 18/2 CRI (Table IV). To simultaneously detect the CRI and Ig isotype of anti-dsDNA Abs, the design of the inhibition assays exploited the ability of these particular antiidiotypic Abs to inhibit the interaction between the autoAb and the Ag, dsDNA, indicating that each anti-CRI binds to a V region site at or near the DNA binding paratope. In these studies, the mAb B6 inhibited the IgG dsDNA binding in all six of the sera containing the corresponding idiotype (10–44%). The mAb λ IIIa inhibited the IgG dsDNA activity of two of the four sera positive for this idiotype (21 and 47%). Ra504 inhibited IgG dsDNA binding of 3 of the 10 sera associated with the 18/2 idiotype (11–14%). Isotype controls did not cause significant inhibition.

Inhibition of IgM anti-dsDNA autoAb activity by the anti-Id demonstrated many cases of overlapping patterns of idiotypic expression between the IgM- and IgG-anti-dsDNA Abs (Table IV). However, for one SLE patient the λ IIIa idiotype, and for two patients the B6 idiotype, were only expressed on the IgG anti-dsDNA Abs. For three patients (which included the SI donor) the 18/2 idiotype inhibition was detected only on the IgG anti-dsDNA Abs, while in two other SLE patients inhibition was detected only in the IgM anti-dsDNA Abs. The common association of an idiotype with $\geq 10\%$ of this autoAb activity suggests that these responses are oligoclonal, and that antiidiotype recognition is not generally de-

stroyed on IgG Abs by Ab V gene hypermutation. The examples of discordant idiotype expression on the IgG and IgM anti-dsDNA Abs may be related to B cell clonal shifts that accompany isotype switch in the autoimmune response.

Significantly, there was a complete concordance between the idiotypes expressed on the serum anti-DNA autoAbs of the donors and the monoclonal IgG Fab anti-dsDNA Abs derived from their respective libraries. In specific, the monoclonal IgG autoAbs from the SI lupus library expressed all three CRI as did her serum IgG anti-dsDNA autoAbs. The serum IgG autoAbs from the donor AA were associated with only the B6 and λ IIIa idiotypes and not the 18/2 idiotype, representing the same pattern detected in her cloned autoAbs.

The above described V gene sequence analyses, and the H–L chain-shuffling experiments, suggested that the generation of dsDNA binding activity in Abs with B6 idiotype-associated H chains requires specific pairing with certain λ -type L chains. To determine whether this restrictive H–L chain pairing could be detected within the in vivo responses of SLE patients, we tested the ability of mAb B6 to inhibit κ and λ chain-specific dsDNA binding by serum Abs. In all six SLE sera with IgG anti-dsDNA Abs associated with the B6 idiotype, significant B6 inhibition (10–55%) was detected in the λ chain-associated dsDNA binding (not shown). In contrast, the B6 Ab did not inhibit κ chain-associated dsDNA binding (not shown). The anti-18/2 CRI reagent and isotype controls did not significantly inhibit either κ or λ L chain-associated dsDNA binding (not shown).

To further evaluate whether the patterns of H–L chain pairing in the autoAbs from the libraries are relevant to the in vivo response, we evaluated the inhibitory effect of adding two or three antiidiotypic reagents together to the same serum sample (Table V). Addition of the B6 and 18/2 specific anti-

Table IV. Serologic Evaluation of SLE Patients and Healthy Controls

	IgG anti-dsDNA	IgG anti-ssDNA	B6 CRI			λIIIa CRI			18/2 CRI		
			Reactivity [‡]	IgG Inhib. [§]	IgM Inhib.	Reactivity	IgG Inhib.	IgM Inhib.	Reactivity	IgG Inhib.	IgM Inhib.
	OD	OD	±	%	%	±	%	%	±	%	%
SLE patients											
AA	1.36	1.58	+	20	10	+	47	23	–		
SI	1.84	2.83	+	11	9	+	0	0	+	11	0
101	2.07	2.75	+	44	0	+	0	0	+	0	0
102	1.63	1.95	+	10	0	–			–		
103	1.60	1.86	+	18	0	–			–		
105	1.16	1.37	+	37	13	–			–		
33	1.11	1.35	–			+	21	0	+	14	0
09	1.68	2.05	–			–			+	0	20
19	1.44	1.17	–			–			+	0	0
02	1.33	0.91	–			–			+	0	16
20	1.31	1.76	–			–			+	0	0
28	1.12	1.53	–			–			+	14	0
26	1.11	1.13	–			–			+	0	0
18	0.97	1.26	–			–			+	0	0
16	1.74	1.66	–			–			–		
104	1.47	1.69	–			–			–		
Range	0.97–2.07	0.91–2.83									
Mean±SD	1.43±0.31	1.67±0.53									
Unaffected twin (BB)											
BB	0.48	1.05									
Healthy controls (n = 32)											
Range	0.07–0.68	0.07–0.88									
Mean±SD	0.26±0.16	0.31±0.22									

*The B6 CRI and the 18/2 CRI are present on nonoverlapping subsets of V_H3 Ig. The λIIIa CRI represents a subset of V_λIII Ig. The patients with B6 CRI on their anti-DNA Abs represent OD 0.18–0.81. The patients with λIIIa CRI on their anti-DNA Abs represent OD 0.40–1.30. The patients with 18/2 CRI on their anti-DNA Abs represent OD 0.16–1.00. [‡]Reactivity represents the level of anti-dsDNA antibodies that are identified by each anti-idiotypic antibody. +/– was designated based on whether the OD was 3 SD above the background level for the assays of sera from healthy controls. [§]Inhibition studies were only performed for the sera with CRI reactive anti-dsDNA antibodies by sandwich ELISA (see Methods). For these assays, sera at a fixed dilution were preincubated with saturating concentrations of antiidiotypic Abs, then added to dsDNA-coated wells and later developed with either IgM or IgG-specific horseradish peroxidase tagged reagent, and then developed. Values were determined by comparison with standard curves of serial dilutions of each sera without inhibitor. For λIIIa and B6 anti-idiotypic mAbs, equivalent studies using MOPC21 as the isotype control displayed 0–4% inhibition. For the anti-18/2 CRI, equivalent studies using rabbit IgG as the isotype control displayed 0–2% inhibition. ^{||}Sera from individual SLE patients are indicated by letter or number code.

CRI reagents to serum SI resulted in additive inhibition, suggesting that the V_H-associated B6 idiotype and the V_H-associated 18/2 idiotype are on separate sets of autoAbs molecules. However, when the B6 and λIIIa antiidiotypic Abs were added together to serum of the lupus twin, AA, inhibition was equal

only to the effect of λIIIa alone, which individually caused greater inhibition than the B6 antiidiotypic Ab. This result suggests that a large subset of the serum IgG anti-dsDNA autoAbs of the AA donor coexpress the B6 CRI on H chains paired with L chains that express the λIIIa CRI. Therefore,

Table V. Inhibition of Serum IgG Anti-ds DNA Antibody Activity by Antiidiotypic Antibodies

SLE patient	B6 CRI*	λIIIa CRI	18/2 CRI	B6 CRI + λIIIa CRI	B6 CRI + 18/2 CRI	λIIIa CRI + 18/2 CRI	All three
AA	20%	47%	–	46%	18%	48%	49%
SI	11%	0%	11%	10%	24%	–	27%
101	44%	0%	0%	44%	43%	0%	40%
33	–	21%	14%	23%	14%	24%	33%

*The percent inhibition of IgG anti-dsDNA binding capacity after preincubation with saturating concentrations of antiidiotypic antibodies. Studies were performed only if CRI was detected on anti-dsDNA antibodies by sandwich ELISA (see Methods). Values were determined by comparison with standard curves of serial dilutions of each sera without inhibitor.

these data further support the impression that there is an equivalent pairing of idiotype reactive H and L chains in the serum of the SLE donor, AA, and in the IgG anti-dsDNA Abs from the AA library.

Discussion

In the current report, we have used repertoire cloning methods to sample Abs from *in vivo* lupus autoAb responses. While repertoire cloning strategies have been previously used in several studies to generate high affinity mAbs of a desired specificity, there has been concern regarding the suitability of this approach to investigate *in vivo* responses. A primary issue is the relationship between library-derived Abs and those constituting the response *in vivo*. Two studies in mouse models have attempted to directly evaluate this relationship, but the results led to different conclusions regarding whether the library approach enabled recovery of Abs with the same V_H and V_L genes sampled by the hybridoma approach (39, 40). While direct side-by-side comparisons of hybridomas and combinatorial library studies have not been feasible in humans, studies have documented that repertoire cloning approaches can isolate human Abs with Ag-binding properties identical to those of the donor's serum Abs to infectious pathogens (41, 42) and autoAgs (43, 44). In the current report, anti-dsDNA Abs were obtained from libraries from two lupus patients, and our sequence analyses and idiotype surveys indicate that these recovered autoAbs can use V regions with predominant H–L combinations that reiterate features of the *in vivo* response (discussed further below). In contrast, multiple selection attempts using a suitable control library, a library from an identical twin discordant for disease, failed to recover anti-dsDNA autoAbs, a disease marker. These studies suggest that in this experimental system the *de novo* pairing of H and L chains unrelated to the *in vivo* autoimmune response did not create this high affinity disease-associated autoreactive binding specificity.

Studies of the B cell repertoires of identical twins discordant for lupus provide special opportunities. Due to their common genetic background, the differential representation of Ab genes in the IgG libraries from the twins must reflect somatic variations, presumably arising from different environmental exposures or from stochastic somatic processes such as those involved in V gene rearrangement. As a direct consequence, differences in the composition of these libraries are likely to be due to overrepresentation of certain activated B cells with higher Ig mRNA levels and/or underrepresentation due to hypermutation of Ab gene sequences that are targets for oligonucleotide-mediated amplification. For both of our twins, serologic studies demonstrated unusually high titers of antinuclear Abs, including high levels of IgM and IgG anti-ssDNA Abs (Table IV), suggesting they share an inherited predisposition to autoimmunity. However, other types of autoAbs were differentially expressed. Importantly, the affected twin had diagnostically high levels of IgG anti-dsDNA Abs, while the healthy twin had only Abs primarily to single stranded DNA that can represent “natural autoAbs” common in healthy individuals (45).

Analyses of the V_H gene expression in each twin's IgG repertoire demonstrated nearly identical V_H family distribution, which we speculate is due to a genetically controlled regulation of the composition of the primary repertoire. While the dominance of the larger V_{H1} , V_{H3} , and V_{H4} families agrees with

earlier reports (30, 31, 46), we unexpectedly found that the small (two to three member) V_{H5} family was overexpressed (~37–42%) in the unselected libraries from both twins (47), while the V_{H5} family represented < 1% of the cloned repertoires of healthy adults evaluated with the same cloning methods (16)(unpublished observation) and with other approaches (30, 48, 49).

While a special role for V_{H5} -expressing B cell clones in SLE pathogenesis has not been previously reported, the V_{H5} -derived AD4-18 from the affected twin has the molecular features of a pathogenic autoAb. Of interest, this autoAb has three clustered mutations in two adjacent codons in a FR3 hot spot (28, 50) associated with the target DNA sequence, A/G-G-C/T-A/T (51, 52). Asparagine replacement mutations at this site have also been previously described in an IgM and an IgG₃ murine anti-DNA hybridoma (53, 54) that use V_H J558 rearrangements that are highly homologous to this human V_{H5} gene. A molecular model predicted the direct involvement in DNA binding of this position within the auxiliary Ag binding loop, “HV4”, of J558-encoded autoAbs (33), and experimental mutagenesis studies confirmed that creation of a basic amino acid at this site improved DNA binding affinity (55). These findings argue that targeted somatic hypermutation of a V_{H5} -rearrangement is involved in the creation of disease-associated autoAbs in the SLE-affected twin.

Sequence analyses identified V_H gene segments in the anti-DNA Abs from our lupus libraries (Table I) that have all been previously described in anti-DNA autoAbs obtained by conventional cellular approaches (reviewed in reference 49). During the design of these studies, we had anticipated that, like the V_{H5} -derived AD4-18 Ab, all of the IgG anti-dsDNA autoAbs would display evidence of extensive hypermutation, that reflect affinity maturation and clonal selection in the periphery. Therefore, it was surprising that about half of these cloned autoAbs represented minimally mutated autoAbs, that all derive from distinct V_{H3} genes that have earlier been identified in nonmutated IgM anti-DNA autoAbs from B cell lines (20, 27). The idiotypic data document that related H chains are commonly part of the anti-dsDNA Ab response of many patients with SLE. While these same V_{H3} genes are also highly expressed in healthy individuals (56), equivalent IgG autoAbs could not be obtained from healthy individuals in identical selection experiments, as dsDNA binding activity appears to require specific V-D-J rearrangement patterns. Thus, although specific V-D-J rearrangements are required, extensive hypermutation is not essential for the *in vivo* development of all parental disease-associated autoreactive B cell clones.

The prevalence of these minimally mutated high affinity IgG anti-dsDNA autoAbs in the *in vivo* autoimmune response of patients with aggressive lupus nephritis supports current paradigms that defects in central B cell tolerance directly contribute to the pathogenesis of SLE. In health, a B cell clone with surface membrane expression of one of these autoAbs should be deleted (or energized or receptor modified) before it could reach the periphery (reviewed in reference 57). If an AD4-37-expressing B cell gained access to sites of tissue damage in the periphery, it might act as a highly efficient Ag-presenting cell for the presentation of self-peptides from nucleoproteins (58), which might then recruit nucleoprotein-specific T cell help and expand the autoimmune response in a cascading loss of peripheral tolerance to nucleoproteins and physically associated self determinants (59–61). Speculatively, evi-

dence that near germline V_H3 anti-DNA Abs dominate SLE repertoires could be indirect evidence of the involvement of B cell superantigens in the initiation or perpetuation of autoimmune disease, as we have recently demonstrated that anti-DNA Abs encoded by these same V_H3 gene segments commonly confer high affinity binding specificity for bacterial or endogenous B cell superantigens (15, 16, 62).

Within our chain-shuffling experiments, we discovered that anti-DNA Abs could only be selected from libraries with L chains from the healthy twin if there were pairings with the V_H3 -encoded AD4-37 H chain; similar autoAbs could not be recovered from the library pairing the AD4-37 L chain with diverse H chains from the healthy twin. The results indicate that the H chain in the AD4-37 autoAb from the affected twin dominates the DNA binding specificity. Consistent with evidence of restrictive H-L chain pairing in lupus autoAbs from B cell lines (26, 27), our chain shuffling experiments and serologic surveys of lupus patients also documented that only a limited subset of λ -type L chains can confer dsDNA binding activity to Abs that use H chains like the AD4-37.

In summary, our studies indicate that the phage display repertoire cloning approach can be an efficient and effective means for characterizing disease-associated Ab responses. While the specific Abs recovered by any method are strongly influenced by the selection methods used, the data suggest that the current recovery efforts are aided in part by the preferential representation in the Ab libraries of mRNA from in vivo expanded B cell clones. While not all lupus anti-DNA antibodies are probably directly involved in the pathogenesis of lupus nephritis, future studies may benefit from Ab selection on complex self-Ags, like nucleosomes, that may be the true in vivo autoimmune targets (63) and which may place greater structural limitations for the selection of appropriate Ag binding sites. Alternatively, selection methods that use glomerular sections or glomerular basement membranes, in formats that correlate with serologic markers of disease activity (64), may offer optimal means for the isolation of pathogenic lupus autoantibodies. Coselection with private idiotypes generated from the donor's serum Abs may also further improve the recovery of relevant cloned Abs, and provide greater confidence in the identification of natural H-L chain pairs. Taken together, these methods have the potential to greatly advance our understanding of the pathogenesis of Ab-mediated autoimmune disease.

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